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To cite this article: A S Minin *et al* 2019 *J. Phys.: Conf. Ser.* **1389** 012069

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Application of NMR for quantification of magnetic nanoparticles and development of paper-based assay

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Abstract. H1 NMR relaxometry is a method that is extremely sensitive to the presence of magnetic nanoparticles, which significantly affect the transverse relaxation time of the water proton. Accordingly, the use of magnetic nanoparticles as labels allows detection of even extremely small amounts of the test substance. This paper analyzes the prospects for applying the method of solid-phase NMR-relaxometric determination of biologically active molecules. The nitrocellulose membranes are chosen as a solid phase and nanoparticles based on iron core with a carbon shell are used as magnetic labels. The possibility of detecting small concentrations of magnetic particles in porous medium is demonstrated. Finally, the ability to detect extremely low concentrations of an analyte, in this case, streptavidin protein (0.5 ng/ml to 100 ng/ml), which is actively used in various fields of biology and medicine, is demonstrated.

1. Introduction

Paper-based assays utilizing porous membranes (usually cellulose or nitrocellulose) as solid support allow simple and rapid detection of various analytes: protein markers, DNA, cells, etc [1]. Usually, solid porous support serves as a carrier of biorecognition molecules (antibodies, oligonucleotides) capable of analyte capture. Label producing a signal e.g. color or fluorescent is another important component paper-based sensors. There are various formats of paper-based assays: lateral flow assays (LFIA), microfluidic paper-based analytical devices (μ PADs), flow through assays and immuno-dot blot [2, 3, 4, 5]. Most of such test-system can be used at the point-of-care, thus many of them are non-instrumental, however application of portable and simple measuring devices can greatly increase the versatility of their applications and enhance performance [6, 4].

Magnetic nanoparticles (MNPs) can be used directly for analytical purposes, due to their spontaneous magnetic moment [7]. Also, magnetic nanoparticles can perturb spin-spin relaxation times (T_2) of nearby protons, this property is called r_2 relaxivity. Despite magnetic resonance imaging is the most prevalent application of NMR (nuclear magnetic resonance) relaxation, numerous biosensors underlying this principle were also developed, and, moreover,



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reached the market[8]. In particular, methods based on NMR relaxometry are being developed. Basically they rely on the formation of clusters of nanoparticles or on their desegregation upon the addition of analyte. Clusters have lower relaxivity in comparison with single particles, therefore the change of T2 proportional to analyte concentration occurs [9].

As a rule, nanoparticles in suspension tend to agglomerate [10, 11]. Agglomeration of magnetic nanoparticles is usually enhanced in a magnetic field, which complicates their quantitative NMR relaxometric detection. When using porous membranes with particles bound to them, this undesirable factor is eliminated.

As a convenient model for analysis, we chose a pair of biotin-streptavidin, where we would measure the concentration of streptavidin using a competitive method using paper-based NMR relaxometry. The bond between streptavidin and biotin is one of the strongest noncovalent interactions in nature. Streptavidin and biotin found multiple applications in biosensors, tissue engineering, and drug delivery [12], therefore streptavidin is a simple and convenient model for demonstration of a practical applicability of NMR assay on a porous support.

2. Materials and Methods

2.1. Magnetic nanoparticles synthesis

Iron nanoparticles coated with a carbon shell (Fe@C) were chosen as a magnetic label. These particles were synthesized by the method of levitational-jet synthesis [13, 14]. Briefly, the method consists in vaporizing an iron seed in a stream of a mixture of argon and butane. The condensation of vapors in the same carrier gas leads to the formation of particles with the structure of a metal nucleus-carbon shell.

2.2. PEGylation of nanoparticles

To obtain stable Fe@C suspensions, the DSPE-PEG-2000 (Avanti Polar Lipids, USA) compound were used as a stabilizer. The suspensions were prepared using the method of film hydration followed by ultrasound treatment [15]. A portion of nanoparticles (approximately 100 mg) was placed in a polyethylene tube and 2 ml of chloroform was added to them. Then, 300 mg of DSPE-PEG-2000 were added to the nanoparticle suspension, and the liquid was intensively processed using an ultrasonic activator with an immersion probe. After that, the liquid was evaporated while rotating the tube in the air until a uniform thin film was formed on the walls. To obtain a stable suspension of pegylated nanoparticles, distilled water was added to the tube, followed by intensive sonication. The resulting nanoparticles are designated Fe@C-PEG.

2.3. Modification of nanoparticles with streptavidin

To modify the surface of metal-carbon nanoparticles with streptavidin proteins they were firstly modified by amino ($-NH_2$) groups using aryl diazonium salts, according to the method described in more detail in [16]. And after that, they were coated with BSA protein, cross-linked with glutaraldehyde, which were modified by streptavidin at the next stage. In more detail, the procedure was described in the article [17]. The resulting nanoparticles are designated Fe@C-St.

2.4. Membrane preparation for relaxometric studies

We used commercially available membranes manufactured by Merck-Millipore. These membranes consisted of a mixed cellulose ester with a pore diameter of 3 μm (SSWP29325), 5 μm (SMWP29325) and 8 μm (SCWP29325).

To saturate membrane surface with the magnetic nanoparticles, the membrane was wetted with Fe@C-PEG suspension, dried and washed out with Tween 20 solution. Before T2 measuring of membranes were wetted again with Tween 20 solution.

2.5. Electron microscope examination

For study using SEM, the membranes were saturated with magnetic nanoparticles, as described in the section above (2.4). The control sample of the membrane was treated with all appropriate solutions, without the addition of nanoparticles, and was washed accordingly.

Electron microscope studies of the membrane were performed using a Zeiss Auriga microscope in SEM mode using an in lens detector. Before the examination on the membrane conductive film (an alloy of Au/Pd) was deposited.

2.6. Control of the binding of nanoparticles to the membrane

To evaluate the binding of nanoparticles, membrane samples of size 10x8 mm were wetted with 10 μ l of a suspension with a concentration of Fe@C 0.1 mg/ml. The membranes were dried at room temperature for 24 hours, then each sample was washed in a plastic test tube with 1 ml of Tween-20 aqueous solution (0.1%). The liquid was removed using a mechanical pipette. The concentration of nanoparticles in the washout was controlled by NMR relaxometry according to the procedure described elsewhere [18].

2.7. SQUID examination of membranes

To confirm the reliability of the results of determining the amount of nanoparticles fixed on porous membranes using NMR relaxometry, we compared these results with results based on traditional magnetic measurements. For this, membranes with different amounts of Fe@C nanoparticles were prepared. After wetting with the nanoparticles suspensions, washing, drying, and re-soaking with water, the membranes were investigated using a relaxometer and then dried again. After drying, we measured the magnetization curves at room temperature using a SQUID magnetometer in fields up to 60 kOe. Membranes without magnetic nanoparticles were also measured. The magnetic properties of the membrane are determined by the diamagnetic contribution of the membrane material and the ferromagnetic contribution from nanoparticles. The ferromagnetic contribution to the magnetic moment of the membrane was determined by extrapolating the linear part of the magnetization curve in high field to zero field. The particle quantity was determined by dividing this value by the specific magnetization Fe@C nanoparticles.

2.8. Measuring Relaxation Time T_2 in a Membrane

The Carr-Purcell-Meiboom-Gill (CPMG) sequence was used in experiments to measure the T_2 spin-spin relaxation time of water protons. The measurements were performed at a frequency of 8 MHz and with the time between echoes $TE=0.5$ ms, using a portable, permanent magnets based, relaxometer constructed at the IMP UB RAS with specialized measurement cell for thin (about 0.3 mm in thickness) samples with flat coil (10 mm x 10 mm x 1 mm) .

Inversion of the relaxation curves gave the function of relaxation times distribution. Each relaxation curve $R(t)$ was approximated with the sum of exponents;

$$R(t) = \sum_{i=1}^N A_i \cdot e^{-t/T_{2i}} + \epsilon_i \quad (1)$$

where ϵ_i is the noise. To determine the amplitudes A_i , an algorithm of nonnegative least squares (NNLS) was applied [19], which allows the A_i coefficients and the T_{2i} values to be found.

2.9. Quantitative measurement of streptavidin concentration

Firstly 8 μ m membranes (Millipore, SCWP09025) was biotinylated by sorption of BSA modified with biotin (Bi-BSA). In more detail, the technique of BSA biotinylation of and its modification

of membranes is described in [20]. After that, the membranes were cut into smaller pieces (6 x 10 mm), required for a single measurement. The resulting membranes are designated M-Bi.

Quantitative determination of streptavidin in solution was carried out using a competitive method. M-Bi membranes were placed in streptavidin solutions of various concentrations in PBS and maintained for 30 minutes. Then washed with Tween20/PBS solution and placed in a solution of PBS/BSA/Tween20 for 15 minutes, then washed with Tween20/PBS. Next, the membranes were placed in a suspension of Fe@C-St nanoparticles for 1 hour, after which they were washed with PBS-Tw and the proton relaxation time was measured. The measurements were carried out in the range of streptavidin concentrations from 0.5 to 10,000 ng/ml. All assays were done in triplicate.

3. Results and discussion

3.1. Nanoparticles characterization

According to TEM size of the core of Fe@C nanoparticles is about 7-8 nm, and the thickness of the carbon shell is about 3 nm [21]. The specific magnetization of Fe@C particles reaches to 100 emu/g [14]. After protein modification, these nanoparticles form a stable suspension with an average size of agglomerate about 150 nm (PDI 0.18) zeta-potential approximately -30 mV and high relaxivity[20].

3.2. Determination of concentration of magnetic NPs using NMR relaxometry

Nanoparticles in porous membranes can be either suspended in the fluid filling the pores or fixed on the walls of the pores. The value of T2 depends on the local magnetic field gradient, which in both cases is created by the MNPs [22]. It should be expected that in the case of nanoparticles fixed on the walls of the pores the T2 value should exceed the value observed for the particles dispersed in the pore volume, because of increasing the volume of liquid where the local magnetic field gradient is smaller. This is proved by the measurements results shown in Figure. 1.

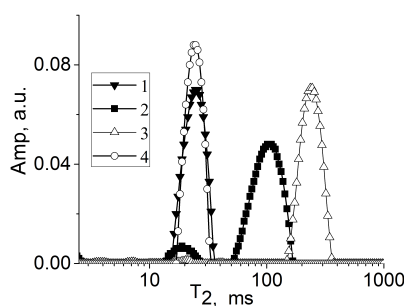


Figure 1. Curves 1, 2 and 3 - distribution of relaxation times depending on membrane treatment: 1- membrane saturated with MNPs suspension (variant 1); 2-membrane impregnated with MNP suspension, dried and then saturated with water (variant 2), 3- membrane saturated with distilled water. 4 - MNP suspension in a free volume. MNPs concentration was 16 ng/ml.

It can be seen that in the first variant the relaxation time T2 coincides with the relaxation time of suspension in the free volume, i.e., the relaxation time is determined only by the magnetic field gradient created by the MNPs in the volume of liquid, and the pore surface does not affect the value of T2. In the second variant, T2 value appeared to be larger than for the first variant.

Further, we determined the range of measurable concentrations of particles on the membranes. The membranes were saturated with 10 μ l of Fe@C-PEG suspension at various concentrations ranging from 0.1 to 0.0004 mg/ml; then the membranes were dried, wetted with a water solution of Tween 20 and placed into a measuring cell. The T2 distributions are shown in Figure ?? for a concentration of 0.1 mg/ml as an example.

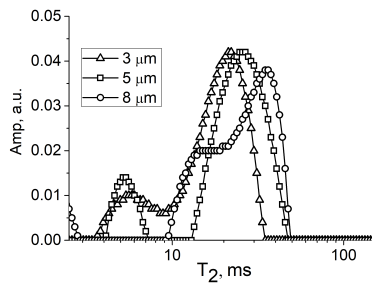


Figure 2. Relaxation time distribution for membranes having different pore sizes as Fe@C-PEG nanoparticles are fixed from the suspension at a concentration of 0.1 mg/ml.).

The presence of several peaks in the distribution may be due to the non-uniform distribution of particles in the porous medium. It is related to the interaction of particles with the membrane surface, to the motion of liquid in the processes of saturation and drying, etc. The narrowest distribution of the relaxation time is observed for the membranes having the pore size at 8 μm , which is likely to be caused by the possibility of a more uniform distribution of particles on such membrane during saturation.

A membrane can be represented as a three-dimensional net with the mesh size distribution. The pore size, as provided by the membrane producer, determines the main size of the mesh corresponding to the size distribution maximum. As the membrane is filled with water, the relaxation time T_2 is determined with the mesh size and spin diffusion. The effect of mesh size on T_2 is averaged over the time T_E , hence a single T_2 is observed. As the relaxation time is measured in the MNPs suspension, the local magnetic field gradients in the vicinity of the particles cause the T_2 to decrease. However, diffusion of water molecules and thermal motion of the magnetic nanoparticles result in an averaged effect of the local gradients [23]. As a result, a single T_2 is detected in the suspensions. In the membrane filled with the MNPs suspension, the particles may be distributed non-uniformly. In the regions of the membrane with elevated MNPs concentration, the local magnetic field gradients are greater and hence can shorten T_2 . In this case, the particles are fixed with the membrane net, as a result, no averaging of T_2 over the entire measured volume occurs during the time T_E , and so a set of T_2 values are detected. However, despite the small local maxima observed on the T_2 distribution pattern, according to the subsequent measurements, the arithmetic weight averaging of T_2 (T_{2a}) is a clear indicator allowing the MNP concentration to be measured in the membrane.

After drying, the MNPs sorbed on the walls of the pores, while re-wetting, they are not washed off, as was confirmed by scanning electron microscopy (Figure 3) and by the methods described in section 2.6. The minimum detection limit for magnetic nanoparticles for this method is 20 pg/ml (which is 2% of nanoparticles contained in the membrane), and no nanoparticles were detected in the washout within the sensitivity range of the method.

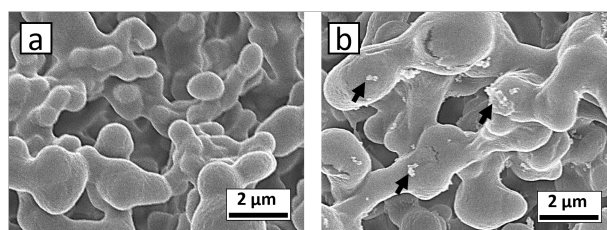


Figure 3. a - membrane with a pore size of 8 μm without Fe@C particles. b - membrane with Fe@C particle aggregates (denoted with arrows).

The dependence of the reciprocal of T_2 ($1/T_2$) on the concentration can be fitted with linear

functions ($R^2 > 0.99$), provided that all range is divided into two concentration areas: the low concentration range from 0.04 ng/cm^2 up to 0.8 ng/cm^2 and the high concentration range from 0.8 ng/cm^2 up to 12 ng/cm^2 (Figure 4).

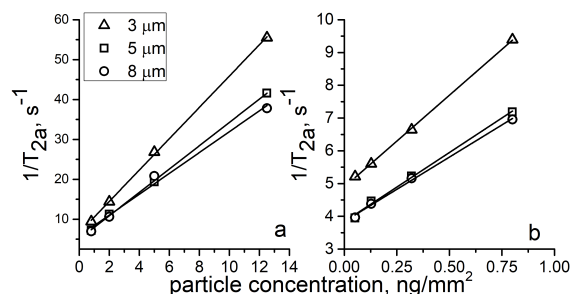


Figure 4. The inverse relaxation time ($1/T_{2a}$) as a function of number of particle concentration shown for membranes having different pore sizes. a high concentration range, b low concentration range

The result of NMR measurements of the concentration of magnetic nanoparticles in the membrane was verified using SQUID magnetometry (Figure 5), the obtained data on the magnetic moment were recalculated in the concentration of nanoparticles per area unit. In the concentration range up to 4 ng/mm^2 the discrepancy between the SQUID and NMR data is no more than 5%, and in the range up to 16 ng/mm^2 no more than 10%.

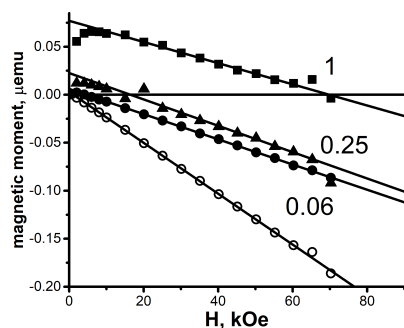


Figure 5. Magnetization curves of membranes containing according to application conditions 1, 0.25, and $0.0625 \mu\text{g}$ of Fe@C nanoparticles, as well as the magnetization curve of two membranes of the same size without magnetic particles (open symbols).

3.3. Determination of concentration streptavidin by NMR paper-based analysis

To test the method for determining the concentration of streptavidin, calibration measurements were performed. At the first stage, the membranes were impregnated with a solution of streptavidin of different known concentrations. After washing, the membranes were impregnated with a suspension containing particles Fe@C-St, the greater the concentration of streptavidin in the calibration solution, the fewer particles were fixed on the membrane (figure) which corresponded to smaller values of $1/T_2$.

It can be assumed that the maximum detectable protein concentration is limited by the number of binding sites for streptavidin on the membrane. In practical terms, this problem is of low relevance and, moreover, is easily solved by diluting the sample under study. The minimum detectable concentration of streptavidin is limited by the relaxation time of the membrane, the binding sites of which are completely filled with magnetic nanoparticles. In this case, the linear plot for determining the concentration of streptavidin by a competitive method ranges from 0.5 ng/ml to 100 ng/ml . Further expansion of the sensitivity limits of the method can be achieved

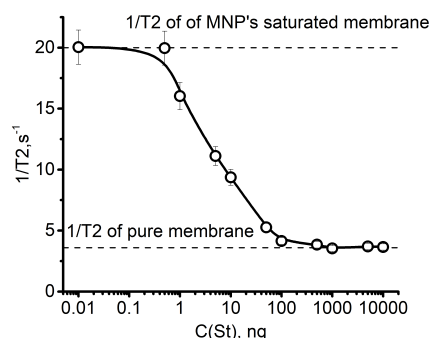


Figure 6. The dependence of the value of $1/T_2$ on the concentration of streptavidin.

through the use of magnetic nanoparticles with greater relaxivity, increasing this parameter will allow detecting nanoparticles in smaller quantities, which, in the case of a competitive measurement technique, will increase the maximum detectable concentration. Another direction of optimization is the use of an NMR relaxometer with a higher frequency, which will allow detection of small T_2 values with a higher resolution.

4. Conclusions

The linear behavior of $1/T_2$ as a function of particle concentration is observed for all the three types of membranes used with the pore sizes of 3, 5 and 8 μm . The narrowest distribution of relaxation times is observed for the membranes with the pore size of 8 μm and such membranes seem to be best suited for the task of determining the magnetic particle concentration on a membrane.

Measurements of the T_2 relaxation time for water protons after fixing magnetic nanoparticles from aqueous suspensions in porous membranes allow the particle concentration to be determined in the range from 0.1 ng/mm^2 to 12 ng/mm^2 of the membrane.

The applicability of the method for the quantitative determination of streptavidin protein using solid-phase NMR detection was shown. It was demonstrated that the detection of this protein is possible within concentrations from 0.5 ng/ml to 100 ng/ml .

Such a high sensitivity allows us to suggest that the NMR relaxometry method in this arrangement may be of interest for developing on its basis a portable device for the diagnosis of various diseases.

Acknowledgments

The work was carried out within the Russian Science Foundation project 17-15-01116. The equipment of the Ural Center for Shared Use Modern nanotechnology UrFU was used.

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